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AN OVERVIEW OF PURIFICATION STRATEGIES FOR MICROBIAL MANNANASES

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ABSTRACT

β-Mannanases hydrolyze mannan-based hemicelluloses and liberate short β-1,4 manno oligomers, which can be further hydrolysed to mannose by β-mannosidases. Such enzymes are not only of academic interest but also they have potential biotechnological applications in a wide range of industrial enzyme market, including food and feed technology, coffee extraction, bioethanol production, slime control agents, pharmaceutical field, pulp and paper industry, etc. Purified mannanases are required for some of the industrial applications like food and pharmaceutical industry. Moreover, purification of mannanase has enabled their successful sequence determination and their three-dimensional structure leading to better understanding of kinetic mechanism. Mannanases from a large number of bacteria, fungi, and actinomycetes have been purified to homogeneity. This article presents a critical review of different strategies which have been employed for the purification of bacteria, fungi and actinomycetes mannanases. Since protein purification is normally done in series of sequential steps involving a combination of different techniques, the effect of a sequence of steps and the number of times each step is used has been analyzed. This will prove to be of immense help while planning mannanase purification. Moreover special features of this class of enzymes, such as carbohydrate binding domains (CBDs) and their importance in the development of affinity methodologies to increase and facilitate mannanase purification has also been discussed. New directions to improve mannanase separation and purification from fermentation media are described.

KEYWORDS : Mannanase, Precipitation, Electrophoresis, Characterization, Molecular Weight, Carbohydrate Binding Domains.

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1. INTRODUCTION

In recent years, hemicellulases have emerged as key enzymes in the rapidly growing biotechnology industry, owing to their multifaceted properties, which find use in a wide array of industrial applications. Mannans are major constituents of hemicelluloses fraction in softwoods and show widespread distribution in plant tissues. The major mannan-degrading enzymes are β-mannanases (E.C 3.2.1.78), β-mannosidases (E.C 3.2.1.25) and β-glucosidases (E.C 3.2.1.21). In addition to these, other enzyme such as α-galactosidases and acetyl mannan esterases, are required to side chain substituents[1,2]. Out of these, most important enzyme is β-mannanase, which hydrolyze mannan-based hemicelluloses and liberate short β-1,4 manno oligomers, which can be further hydrolysed to mannose by β-mannosidases. The β-mannanases are known to be produced by a variety of bacteria, fungi, actinomycetes, plants, animals and have potential biotechnological applications in a wide range of industrial enzyme market, including food and feed technology, coffee extraction, bioethanol production, slime control agents, pharmaceutical field, pulp and paper industry etc.[2,3].

Purified mannanases are required for some of the commercial applications like bioconversion of mannan into a commercially usable form of manno-oligosaccharides and mannose which can be used for pharmaceuticals, food and in improvement of animal feeds etc.[2,3]. In addition, purified mannanase are required for determination of their primary amino acid sequence and three-dimensional structure. Knowledge of the three-dimensional structure of mannanases plays an important role in designing and engineering mannanases for specific purposes. The X-ray studies of pure mannanases enable the establishment of structure–function relationships for a better understanding of the kinetic mechanisms of mannanase action on hydrolysis, identification of catalytic residues and binding residues of oligosaccharides and also investigate the basis of adaptation mechanism of β-mannanase to extreme conditions like thermo-alkali-stability[4-8].

Industries look for purification strategies that are inexpensive, rapid, high yielding and amenable to large-scale operations. Various traditional purification techniques such as precipitation and chromatographic methods etc. can be used for the purification of mannanases. However, main constraint in traditional purification strategies is low yield. Alternative new technologies such as aqueous two-phase systems and affinity methods (using Carbohydrate Binding Domains) are gradually coming to the forefront in the purification of mannanases. Purification protocols available in literature are important for consultation when attempting to purify any new preparation. So far there is no review available which gives complete information regarding purification of mannanases. Thus, a review article summarizing up-to-date literature on purification of mannanases shall serve as a ready reference for researchers engaged in the area of mannanase purification. The present review summarizes the various purification strategies applied by different workers for purification of mannanases. The extent of purification varies with different protocols; a comparison of strategies used by various workers has been discussed in this article. On analyzing various articles for purification microbial mannanases published from 1976 to 2013, conclusions have been drawn to develop the best strategy used in the purification of mannanases. Moreover special features of this class of enzymes, such as carbohydrate binding domains (CBDs) and their importance in the development of affinity methodologies to increase and facilitate mannanase purification has also been discussed.

2. OVERALL ANALYSIS OF MANNANASE PURIFICATION METHOD

Most of the microbial mannanases are extracellular and purification process is usually followed by the removal of cells from culture broth, either by centrifugation or by filtration.

The enzyme from cell- free culture broth is then concentrated by ammonium sulphate precipitation or extraction with organic solvents. Further purification is done by a combination of several chromatographic methods. The data has been complied and shown in Table 1. This table summarizes the purification strategies used for each enzyme, including the number of steps in purification scheme (which includes the concentration and purification steps) and the corresponding recovery yields, purification factor, and final specific activities of the different enzymes.

Table 1
Purification strategies for microbial mannanases

S.No.	Microorganisms	Source	Temp. optima of activity	pH optima of activity	Purification strategies	No. of steps	Recovery (%) / Purification fold	Specific Activity	Molecular weight	References
Bacteria										
1	*Bacillus* sp. KK01 Four enzyme component F1 F2 F3 F4	Soil	 60 55 55 50	 7.1 7.1 7.1 7.1	Isopropanol precipitation (30-65%), Diethylaminoethyl (DEAE)-ion exchange chromatography	2	 9.7/33.5 1.7/28.5 9.7/22.5 8.9/32.5	 1.3 1.1 0.9 1.3	NR	(56)
2	*Bacillus* sp.TN-31	Soil	50	6	Poly ethylene glycol, Ammonium sulphate precipitation (30-60%), DEAE-cellulose chromatography, Hydroxylapatite chromatography, Sephadex G-200 chromatography	5	12/246	56	131 kDa	(37)
3	*Bacillus* sp. AM-001 M-1 M-11 M-111		60 60 65	9.0 9.0 8.5	Ammonium sulphate precipitation (80%), DEAE-Toypearl 650 M column chromatography, Hydroxyapatite column chromatography, Sephacryl S-200 column chromatography	4	31/13.6 9/20.4	312 470	58 kDa 59 kDa 42 kDa	(24)
4	*Bacillus stearothermophilus*	Culture Collection	70	6.5	Ammonium sulphate precipitation (50-95%), Sephacryl S-200 Gel filtration, Mono S cation exchange chromatography, Phenyl-superose hydrophobic interaction chromatography, Gel filtration	5	4.5/43	97	162 kDa (dimeric) Native-PAGE	(11)
5	*Bacillus subtilis* KU-1	Culture Collection	50-55	7.0	Ammonium sulphate precipitation, DEAE-Toyopearl, Phenyl-Sepharose, FPLC Mono Q column chromatography	4	39/810	407.7	39 kDa	(57)
6	*Bacillus licheniformis*	Culture Collection	60	7.0	Flocculation,Ultrafiltration (50 kDa), Ultrafiltration (10 kDa), DEAE-cellulose column (1^{st}), DEAE-cellulose column (2^{nd})	5	47/33.1	4341	NR	(58)
7	*Bacillus subtilis* SA-22	NR	70	6.5	Ammonium sulphate precipitation, Hydroxyapatite chromatography, Sephadex G-75 gel filtration, DEAE-52 anion exchange chromatography	4	23.43/30.75	34780.56	38 kDa	(59)
8	*Bacillus* sp. JAMB-750	Soil	55	10	Ammonium sulphate precipitation (30-60%), DEAE-Toyopearl ion exchange chromatography, Hydroxyapatite chromatography	3	9.0/454	39.5	130 kDa	(60)
9	*Bacillus subtilis* WY34	Soil	65	6.0	Ammonium sulphate precipitation (40-80%), Superdex column,Q-Sepharose fast flow anion exchange column chromatography	3	20.3/5.4	8302.4	39.6 kDa	(53)
10	*Bacillus subtilis* B36	NR	50	6.4	Ammonium sulphate precipitation (30-80%), Anion exchange (HiTrap Q-Sepharose column,Gel filtration Superdex S-200 chromatography	3	7/178.43	927.84	38 kDa	(61)
11	*Bacillus* sp. MSJ-5	Konjac field	50	5.5	Ammonium sulphate precipitation (40-60%), Bio-gel P60 gel filtration, DEAE-Sepharose anion exchange chromatography	3	18.9/19	5383	40.5 kDa	(62)
12	*Bacillus* sp. MG-33	Desert soil	65	6.5	Ammonium sulphate precipitation (50%), Sephadex G-150 Column chromatography	2	16/11.8	591.7	NR	(13)
13	*Bacillus circulans* M-21	Soil	50	7.0	Acetone precipitation,Q-Sepharose fast flow anion exchange column chromatography	2	70.1/9.0	19373.3	33.4 kDa	(10)
14	*Bacillus subtilis* BCC 41051	Soil, Manure	60	7.0	Ultrafiltration (10 kDa), DEAE-Sepharose CL-6B column chromatography, Phenyl Sepharose CL-4B column	4	3/94	3169	38 kDa	(23)

		samples			chromatography, Gel filtration Superdex S-200 chromatography					
15	*Bacteroides ovatus* 0038-1	Culture Collection	37	6.5	Mono Q ion exchange chromatography, Gel filtration on Bio-Gel P-10 column	2	NR	76	94 kDa/ 61 kDa/ 43 kDa	(41)
16	*Bacteroides ovatus* 0038-1	Culture Collection	37	6.5	Ultrafiltration, DEAE-Sephacel chromatography, Chromatofocusing, Gel filtration	4	4/138	NR	61 kD	(22)
17	*Clostridium butyricum* / *beijerinckii*	Human feces	50	7-8	Ultrafiltration (10 kDa), Ammonium sulphate precipitation (35%), DEAE-Toypearl, Phenyl Superose HR	4	2.32/752	3.761	53 kDa	(63)
18	*Clostridium tertium* KT-5A	Soil	55	6.5	Ammonium sulphate precipitation (50-90%), DEAE-Sephadex A-50 anion exchange column chromatography, Sephadex G-100 gel filtration	3	NR	NR	NR	(64)
19	*Cellulosimicrobium* sp. strain HY-13	Soil	50	7.0	Ammonium sulphate precipitation (40-80%), HiPrep column chromatography, HiPrep DEAE resin fast flow column, Superdex 200 pg column, Gel permeation chromatography	5	71.2/48.1	NR	34.9 kDa	(15)
20	*Flavobacterium* sp.	Culture collection	35	6.0	Ultrafiltration	1	NR	NR	46 kDa	(65)
21	*Paenibacillus* sp. DZ3	Soil	60	5.0	Ammonium sulphate precipitation (80%), DEAE ion exchange chromatography, Phenyl Sepharose, Hi-Trap phenyl sepharose chromatography (HIC)	4	15/34	169	39 kDa	(66)
22	*Paenibacillus cookii*	Soil	50	5.0	Ammonium sulphate (40-60%), DEAE-Sepharose, 1st Sephacryl S-100 HR, 2nd Sephacryl S-100 HR	4	6.4/90.2	635.4	68 kDa	(16)
23	*Thermotoga neapolitina* 5068	Culture Collection	90-92	6.9	Ammonium sulphate precipitation, Butyl-sepharose hydrophobic interaction chromatography, Mono-Q anion exchange column chromatography	3	13.6/4.8		65 kDa	(9)
24	*Vibrio* sp. strain MA-138	Seaweed	40	6.5	Ammonium sulphate precipitation (75%), Sepharose-Q fast flow, Toyopearl HW-55S column, Gigapite column, Mono Q column	5	23/633	51.9	49 kDa	(28)
Fungi										
25	*Aspergillus niger*	Wood Sample	30	5.5	Ammonium sulphate precipitation, Gel filtration, Ion exchange chromatography	3	23.1/15.89	19.09	NR	(67)
26	*Aspergillus tamarii*	Culture Collection	37	4.5	Poly ethylene glycol, Hydroxyapatite column, DEAE-cellulose column chromatography	3	8.3/9.0	26	53 kDa	(25)
27	*Aspergillus niger*	Culture Collection	50	3.5	Ammonium sulphate precipitation, Anion-exchange chromatography (Resource Q 1-ml anion-exchange column), Gel filtration (HiLoad 16:60 pre-packed with Superdex 200)	3	46/46	3860	40 kDa	(68)
28	*Aspergillus oryzae* & *Aspergillus niger*	NR	40	6.0	Acetone precipitation, DEAE-cellulose column chromatography, SP-Trisacryl M (sulphopropyl-poly [N-tris (hydroxy-methyl)methyl acrylamide] cation exchange chromatography, Sephadex G-10 column chromatography	4	10.2/90.7	1760	110 kDa	(12)
29	*Aspergillus fumigatus* IMI 385708 Man 1 Man 11	Culture Collection	60	4.5	Ultrafiltration (10 kDa), DEAE Sepharose chromatography, Phenyl Sepharose chromatography Ultrafiltration (10 kDa), DEAE Sepharose chromatography, Phenyl Sepharose chromatography	3 3	10.6/102.4 0.53/49.0	471.0 225.4	60 kDa 63 kDa	(31)
30	*Aspergilllus niger gr*	Soil	55	5.5	Ammonium sulphate precipitation (0-80%), DEAE-Sephacel chromatography	2	9.5/7.82	65.06	66 kDa	(18)
31	*Aspergillus oryzae* NRRL 3448	Culture Collection	55	5.5	Acetone precipitation (60%), DEAE-Cellulose anion exchange column chromatography, Sephadex G-100 gel filtration	3	9.4/10	156.8	NR	(69)
32	*Aspergillus niger* LW-1	Soil	65	4.8	Ammonium sulphate precipitation (75%), Phenyl sepharose CL-4B hydrophobic chromatography, DEAE-Sepharose Fast	4	NR		42 kDa	(14)

					Flow ion exchange chromatography and Sephadex G-75 gel filtration.					
33	*Penicillium occitanis* Pol6 (Man 111)	Cayla Company	40	4.0	Ammonium sulphate precipitation (60%), Gel filtration on Biogel P-100, Ion-exchange chromatography on MonoQ-Sepharose column	3	1.87/25.17	129.37	18 kDa	(21)
34	*Sclerotium rolfsii*	NR	74	2.9	Ultrafiltration (10 kDa), Anion exchange chromatography using Q-Sepharose fast flow column, Gel filtration on Superdex 75 column	3	25.85/5.52	475	61.2 kDa	(40)
35	*Scopulariopsis candida* strains LMK004 LMK008	Solar saltern	50 40	5.0 6	Ammonium sulphate precipitation, Q-Anion-exchange chromatography Ammonium sulphate precipitation, Q-Anion-exchange chromatography	2 2	5.99/64.73 3.07/5.67	27864.6 3321	41 kDa 28 kDa	(17)
36	*Trichoderma reesei* I II	NR	70 70	3.5 3.5	Ion exchange chromatography, Affinity chromatography, Chromatofocusing	3	NR	NR	51 kDa 53 kDa	(27)
37	*Trichoderma reesei* C-30	Culture Collection	75	5	Ethanol precipitation, Chromatofocusing, Mono Q anion exchange column chromatography	3	11/18	21.6	46(±2) kDa	(26)
Actinomycetes										
38	*Streptomyces galbus* NR	Soil	40	6.5	Ammonium sulphate precipitation (0-80%)	1	62.79/8.71	44.79	NR	(70)

Analysis shows that microbial mannanases are mainly purified by chromatographic methods, using from two to five purification steps (including precipitation and concentration), and providing recovery yields ranging from 0.53 to 71.2%. As expected, lower yields were generally obtained when greater numbers of purification steps were used. When two purification steps are used, the purification fold can range from 5.67 to 64.73 and with four purification steps it can range from 13.6 to 810. The data also suggest that lower recovery yields are obtained with fungal mannanases in comparison to bacterial mannanases. The relationship between average mannanase yields and the number of purification steps in the overall purification schemes for enzymes is shown in Fig 1. Increasing the number of steps led to decrease in mannanase recovery, and with five purification steps, low enzyme recovery is observed (about 13%). In general, high purification fold correlated with purification schemes for low molecular weight mannanases (ranging from 39 to 53 kDa).

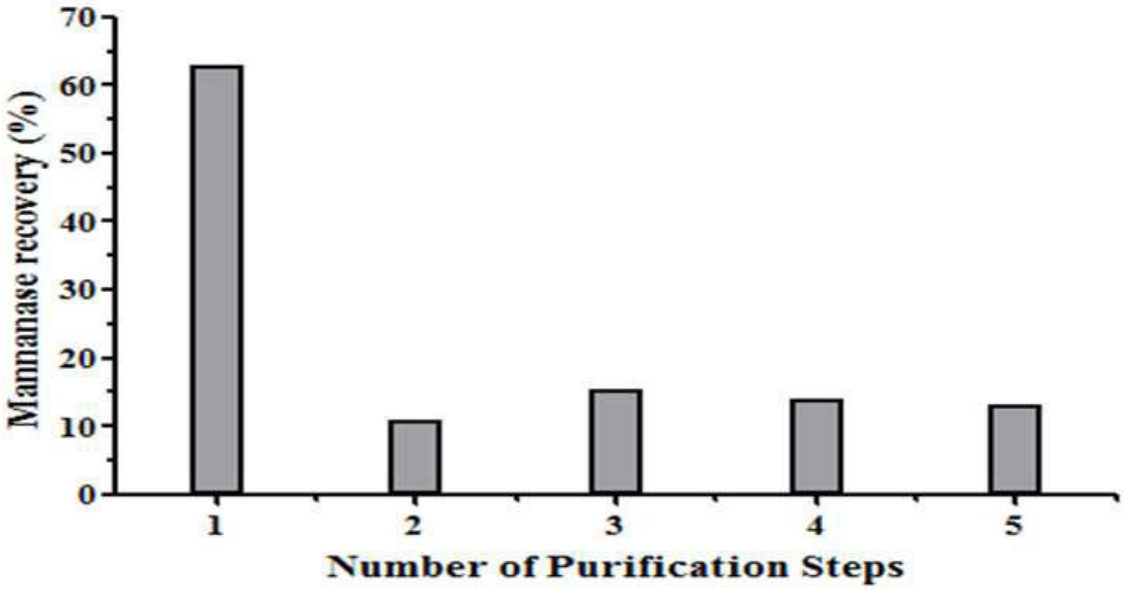

Figure 1
Mannanase yields (averages of 38 purification schemes) as function of the number of steps of purification procedure.

Various mannanase purification schemes were studied in detail, taking into account the sequence of the purification steps, the number of times each purification method is used (Fig 2) and average purification fold achieved with different strategies at each step (Fig 3). In the first step of mannanase purification about 71% of the researchers used precipitation, with 58% of these used ammonium sulphate and 13% used organic solvent (mainly acetone). An alternative to precipitation methods was ultrafiltration which was used as a first step in 18% of the procedures for protein concentration (Fig 2). Maximum average purification fold (6.05) at first step has also been reported when ammonium sulphate was used as first step of purification. With other methods, average purification fold ranged from 5.67 to 1.10 (Fig 3).

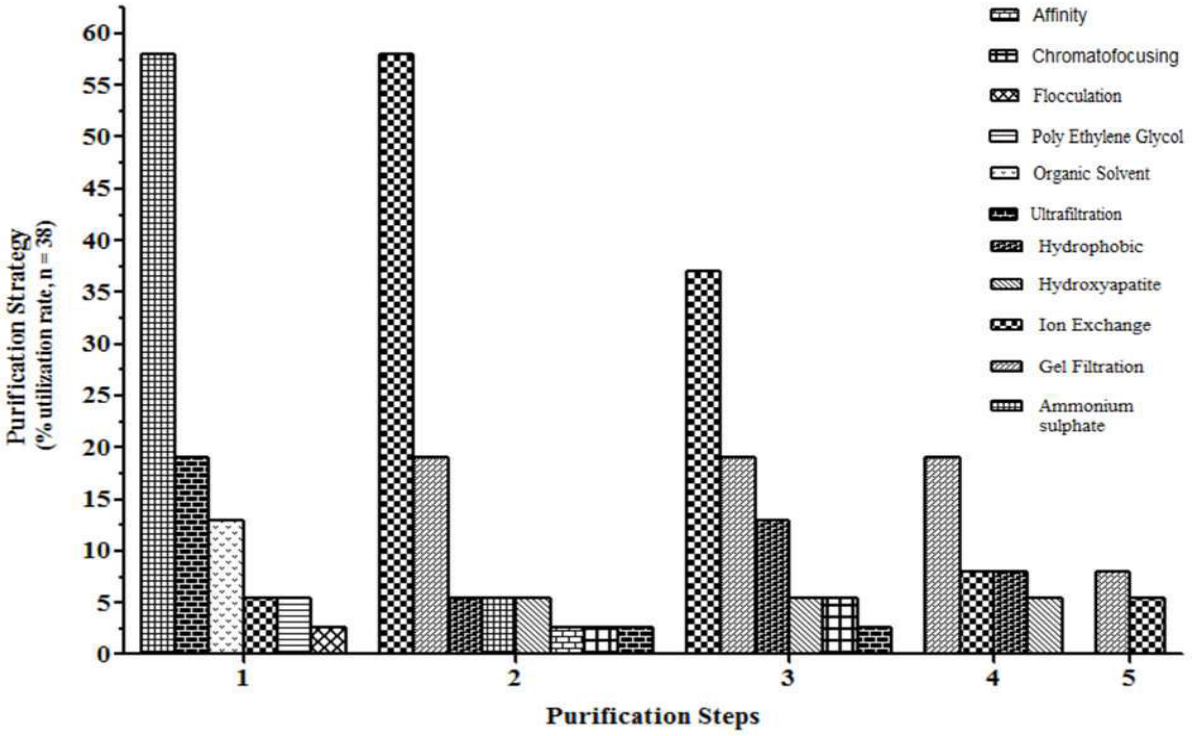

Figure 2
Strategies used in mannanase purification in relation to number of steps, showing different methods used in each step according to their utilization rate

In the second step of mannanase purification, ion-exchange chromatography (IEX) has been used by 58% of the researchers, other methods were used from 18.40% to 2.63% (Fig 2). Maximum average purification fold (47.03) at second step has been reported when IEX was used. With other methods, average purification fold ranged from 9.30 to 3.20 (Fig 3). Resins with weak ion exchange groups were most commonly used, with diethylaminoethyl (DEAE) group being the most commonly used group (60%) in anion-exchange chromatography. Strong ion exchanger compounds were used in 31% of cases with Mono Q, Q-Sepharose, Mono-S and SP-Trisacryl M were the most commonly used columns[9-12].

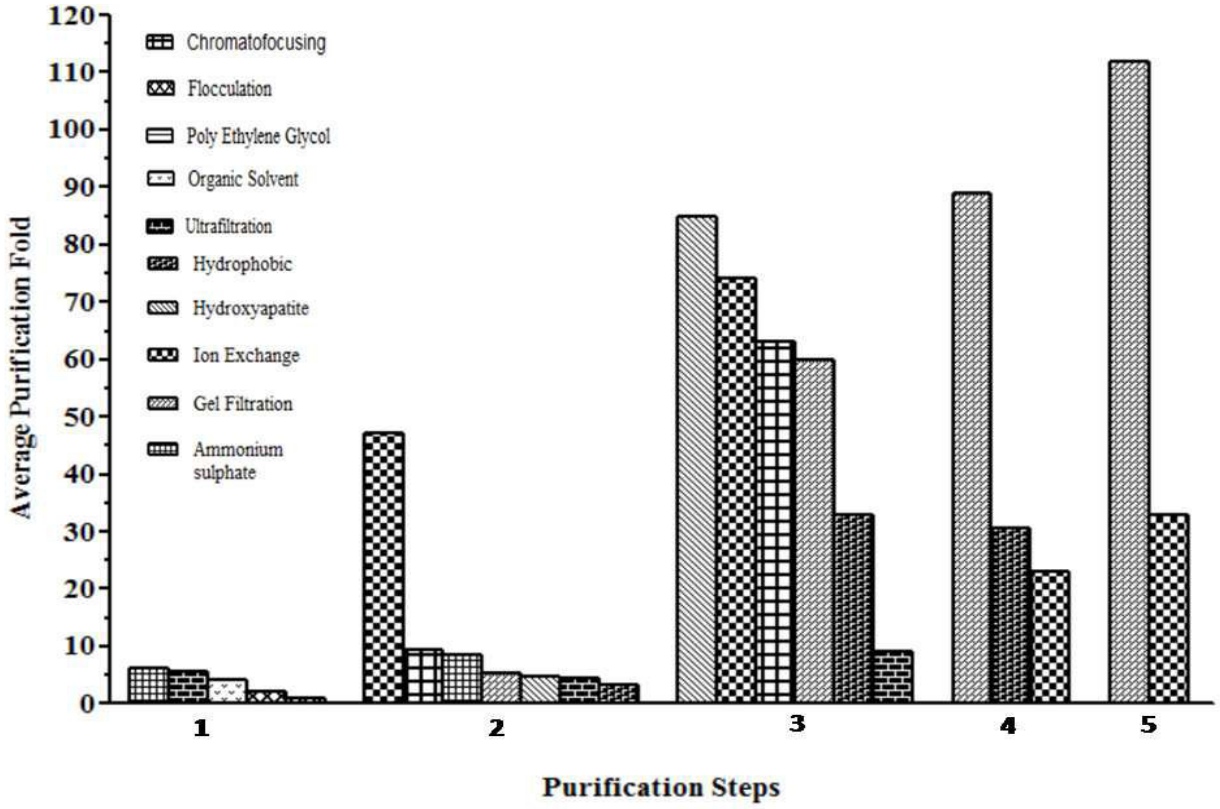

Figure 3
Relationship between average purification fold with different strategies at each step for mannanase purification.

In the third step of mannanase purification ion-exchange chromatography (IEX) has been used by 37% of researchers, other methods were used from 18.40% to 2.63% (Fig 2). Maximum average purification fold (85.33) at third step has been reported with hydroxapatite chromatography. With other methods, average purification fold ranged from 74.02 to 9.2 (Fig 3). In the fourth step of mannanase purification gel filtration (GF) has been used by 18% of the researcher, other methods were used by 7.89% to 5.26% researchers (Fig 2). Maximum average purification fold (89.30) at fourth step has also been reported when GF was used. With other methods, average purification fold ranged from 30.50 to 23.62 (Fig 3). Various highly cross linked gel matrices such as Sephadex, Sephacryl, Superdex and Biogel P are most commonly used[13-16]. A five steps purification procedure was used when a high purity level was required. At fifth step GF was used in 7.8% of the protocols comparatively to IEX (5.2%) (Fig 2). Average purification fold at this step ranged from 112 to 33 (Fig 3). Most of the purification schemes examined, included the use of a three-step strategy (36.8%) for the purification of mannanses giving average purification fold of about 85.33. A four-step and five step strategies were used in 26% and 13% of the reports respectively. On the basis of this analysis, a three-step mannanase purification strategy can be suggested. The data showed that the purification factor was not only related to the purification strategy used, but was also dependent on other factors, such as enzyme origin and excretion level.

3. DRAWBACKS OF PURIFICATION STRATEGIES

Analysis of recovery yields and purification factors achieved per step of mannanase purification was undertaken in order to determine the limiting step of the purification process. Detailed study of the purification

procedures led to the conclusion that, in general, the first step in the purification procedures was the limiting step. Concentration steps involving ammonium sulphate precipitation, organic solvent and ultrafiltration were steps mainly responsible for the low final recovery yields. In 31% of the procedures, decrease in recovery yield ranging from 11 to 69% has been reported. Further analysis of the multiplicity of purification (ratio of the purification factor between steps) obtained after IEX and GF chromatography, showed that the decrease in recovery yield did not correlate with an increase in the purification factor.

3.1 Problem Associated with Some Purification Methodologies

3.1.1 Precipitation

With regard to mannanase purification, the most commonly used salt for precipitation was ammonium sulphate. However this led to average low enzyme recovery yield in 22% cases[13,17-18]. Other reports shows that ammonium sulfate promotes flocculation rather than precipitation of mannanases[19]. In these cases, a decanting funnel can be recommended for visualizing the separation of enzyme phases. Each of the enzyme phases can be analyzed separately in order to balance the enzyme protein concentration and enzyme activity and to check the purity level of the enzyme by electrophoresis or high-pressure liquid chromatography (HPLC). Another problem associated with the utilization of ammonium sulfate precipitation is that salt interferes in determinations of mannanase activity, leading to overestimations of such activity in crude extracts[20]. Thus, in general a dialysis or desalting step is generally required before the determination of enzyme activity. Ultrafiltration membranes can also markedly reduce mannanase recovery yields (MW >20 kDa), owing to the ability of mannanase to pass through ultrafiltration membranes with low-molecular-weight cut off values (5–10 kDa). This drawback was suggested to result from mannanase compact structure and/or from non uniformity in membrane pore size[21]. Since most commercial ultrafiltration membranes are made of cellulose or its derivatives, the presence of carbohydrate binding domains (CBDs) in mannanase should be determined before ultrafiltration. If they contain CBDs, mannanase can be retained on ultrafiltration membranes. Some reports refer to activity losses of about 27-40% after an ultrafiltration step[22,23].

3.1.2 Gel Filtration

GF chromatography is widely used as a mannanase purification method, however mannanase may interact with some GF matrices. The interaction of mannanases with the column matrices Superdex 200[15,23] and Sephacryl S200[11,24] retards mannanase elution. These results could suggest the presence of carbohydrate binding domain (CBD) in mannanase that interact with gel matrix (agarose and dextran), since dextran consist of α-glucose units, whereas cellulose is composed of β-glucose units, resulting in delayed protein elution. Enzyme glycosylation was also described as causing anomalous elution patterns in gel filtration chromatography. Since this feature is common among microbial mannanase especially fungi it would be useful to check whether the enzyme being purified is glycosylated[25-33].

3.1.3Electrophoresis

Some microbial mannanases showed a discrepancy in molecular weight, as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), GF chromatography, and HPLC. Such differences in mannanase molecular weight determinations can in some cases be associated with enzyme glycosylation[33-35]. Interaction of the glycoprotein with the acrylamide matrix in SDSPAGE can lead to anomalous migration of an enzyme in the acrylamide gel. Anomalous migration in SDS-PAGE of proteins with an unusual composition of amino acids, could result from differences in surface net-charge density[11]. Consequently, a gradient gel is necessary to determine molecular weights with accuracy. In substrate gel, mannanase can bind tightly to a substrate and their migration on gel can be delayed, affecting a molecular weight

determination. As an example, a mannanase from *Vibrio* sp. strain MA-138 containing Carbohydrate binding module (CBM) that binds specifically to soluble mannan exhibited slower gel migration owing to interaction with substrate gels[36]. Most microbial mannanases are single-subunit proteins with molecular weights ranging from 18 to 131 kDa[21,37]. However, a strain of *Bacillus stearothermophilus* produced three mannanases denoted as A, B and C. Migration of the mannanase B and C on SDS-PAGE showed identical and relative molecular weights of 73±6 kDa. The mannanase A migrated as two sharp bands on SDS-PAGE, one of which migrated identically to mannanase B and C while the migration of second band corresponded to a protein of lower molecular weight, 58±5[11]. Similarly four different mannanases having molecular weight of 22, 61.2, 57 and 42 kDa, have been reported from *Sclerotium rolfsii*[38]. *Bacteroides ovatus*, a gram negative obligate anaerobe bacteria produce two mannanases, one outer membrane bound and one soluble. It was found that outer membrane bound mannanase was made up of three subunits having MWs 94.5, 61 and 43 kDa respectively,[39] whereas soluble mannanase was monomeric protein having MW 61 kDa on SDS-PAGE[22]. Both enzymes differs each other with respect to stability and isoelectric point. Difference in the gel mobility behavior of mannanases suggests that extra care is needed in analyzing these enzymes with SDS-PAGE gels when their molecular weight and purity are to be evaluated.

4. PURIFICATION OF RECOMBINANT MANNANASES

Gene cloning is a rapidly progressing technology that has been instrumental in improving our understanding of the structure–function relationship of genetic systems. Apart from this purification of recombinant enzyme is straight forward as high expression levels of the target enzyme can be attained[40], which increases the ratio of target enzyme to contaminants, and can eliminate or reduce the requirement for an initial concentration step. Purification can be further simplified if the recombinant protein displays some pronounced physicochemical characteristic, which is not displayed by native host protein. This is exemplified by the recombinant expression of extremely thermostable enzyme (derived from thermophilic bacterium *Thermoanaerbacterium polysaccharolyticum*) in mesophilic host organism such as *E.coli*. Heating of crude extract at 70°C for 10 min results in the denaturation and precipitation of native *E.coli* proteins, which can be removed by centrifugation, leaving behind a partially purified recombinant protein in solution [41]. Genetic engineering techniques also facilitate the incorporation of specific peptides or protein tags to the protein of interest. A tag is chosen which confers on the resultant hybrid protein some pronounced physicochemical characteristics, facilitating the subsequent purification. Such a molecule is normally produced by fusing a DNA sequence which codes for the tag to one end of the genetic information encoding the protein of interest. Tags that allow rapid and straightforward purification of the protein by techniques such as ion exchange, hydrophobic interaction and affinity chromatography have been designed and successfully employed. A number of microbial mannnases have been cloned and expressed in heterologous hosts[2] . In most of the recombinant mannanases a purification tag consisting of polyhistidine are employed to purify protein by metal chelate affinity chromatography. The metal ions most commonly used are Zn^{+2}, Ni^{+2} and Cu^{+2}. Basic groups on protein surfaces, most notably the side chain of histidine residues, are attracted to the metal ions, forming the weak coordinate bonds. Elution of bound proteins is under taken by lowering the buffer pH (this cause protonation of the histidine residues, which are then unable to coordinate with the metal ions)[42-49].

5. CONCLUDING REMARKS AND FUTURE TRENDS ON MANNANASE PURIFICATION STRATEGIES

A critical overview of the purification procedures for microbial mannanases shows that they have largely been purified through traditional multistep chromatographic methods. These are

time-consuming and generally yield low recoveries. An optimal sequence of chromatographic methods that maximizes recovery yields and purification factors is difficult to achieve, but a three-step mannanase purification strategy can be suggested which can give appropriate purification fold and yield (Ammonium Sulphate precipitation Anion Exchange Chromatograph Hydroxyapatite Chromatography/Anion Exchange Chromatography). We found that IEX was commonly used as the first chromatographic step as well as last step along with gel filtration in the purification schemes for these enzymes. A detailed examination of the mannanase purification procedures reported in the literature showed that, in general, the first step of the purification schemes was the limiting step with regard to the yield. Aqueous two-phase systems (ATPS) can be applied to the first downstream purification steps for mannanases, permitting simultaneous separation and concentration of the target protein and the integration of fermentation with downstream processing. We found very few examples of ATPS for mannanase purification in the literature[50,51]. ATPS-based approaches to enzyme recovery may have immense potential in the purification of mannanases. CBDs are specific features of multimeric mannanases that can affect the development of mannanase affinity-purification processes[52]. The CBDs are also versatile new tools for the construction of efficient affinity-purification systems through the engineering of a fusion protein consisting of a target polypeptide and specific CBDs in combination with a specific affinity matrix[53,54,55]. These structures are used as biological anchorage systems and are already commercially available (Velcro®), linking a large variety of molecules and chemicals to readily available, biocompatible cellulose matrices. Velcro (FuturaGene Israel Ltd [formerly known as CBD Technologies, Ltd]) is a registered trademark that uses cellulose-binding domains (CBDs). CBD is a protein, which acts as a biologic "Velcro," binding to cellulose and chitin. The CBD can thus serve as a molecular anchor or trap by binding a wide variety of molecules of interest to readily available cellulose matrices, thus serving a broad range of purposes, including purification, diagnostics, and bioprocessing: plant transgenics; waste management—bioremediation, environmental decontamination; biotechnology—peptide isolation, enzyme immobilization; diagnostics—diagnosis of water and food contamination. Furthermore, plants transformed with the CBD gene show significant growth enhancement.

In order to fully exploit the immense potential of CBDs, it is necessary to understand the ways in which they bind to and desorbs from cellulose. The range of their applications will be greatly extended by having a comprehensive library of CBDs with different binding and desorption characteristics and substrate "anchoring" specificities. Using mannanase CBD for biospecific interaction with cellulose offers several advantages over chemical immobilization of mannanases, as do other protein-engineering methods of coupling proteins to matrices. The characteristics for anchoring CBDs do not require chemical activation of the matrix, thus eliminating the use of hazardous compounds. Purification and immobilization may be provided in a one-step procedure, decreasing the number of steps in the purification scheme, which significantly reduces the downstream costs. Also, cellulose is an inexpensive support and can be obtained commercially in various forms. A hurdle for many current biotechnology processes is the large volume of water from which an enzyme or other desired product must be recovered, thereby requiring a concentration step before purification. The major production costs (50–90%) for a typical biological product reside in the strategy used for purifying. . Therefore, an efficient and cost effective bio separation technique for purifying the protein in high quantity is required. The key to the wide utilization of mannanases lies in furthering the understanding of their downstream processing and integrating it with their upstream processing. This should provide better insights into improving the economics of the entire purification process. To obtain a competitive purification process, it is necessary to evaluate

both the separation and purification processes, taking into account each individual purification step. The influence of each step on the following step, in terms of recovery yield and purification factor is crucial for the optimization of a purification strategy. Process strategies that integrate unit operations must be developed to decrease the total number of steps in the downstream purification process. A multidisciplinary approach, based on techniques from molecular biology to engineering sciences, is needed to more efficiently purify mannanases.

CONFLICT OF INTEREST: None

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